Capillary Electrophoresis Study on the Dimeric SOD Enzyme in the Presence of Ascorbic Acid

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A Notable decrease of the peak intensity of the capillary electrophoregram due to the dimeric SOD molecule was observed when a solution containing copper(II) chloride and ascorbic acid was added to the SOD solution, indicating that the capillary electrophoresis method is useful to detect the dissociation of the dimeric SOD molecule in solution, and that dissociation of the dimeric SOD molecule is induced by the presence of hydrogen peroxide. The present results may give reasonable countermeasures towards the sporadic amyotrophic lateral sclerosis in future.

Key words: Dissociation of Dimeric SOD, Capillary Electrophoresis, Hydrogen Peroxide

Introduction

Cu,Zn superoxide dismutase (Cu,ZnSODs) are metalloenzymes involved in the mechanism of cellular defense against oxidative damage (Fridovich, 1975; Hough and Hasnain, 1999). Eukaryotic Cu,ZnSODs are homodimers that contain one atom of zinc and one atom of copper per subunit and catalyze the dismutation of the superoxide anion at a diffusion-limited rate enhanced by electrostatic guidance of the substrate to the active site (Hough and Hasnain, 1999). Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease, is a neurodegenerative disorder characterized by the destruction of large motor neurons in the spinal cord and brain (Matsumoto and Fridovich, 2002). Approximately 5–10% of cases are familial, and 15–25% of familial ALS (FALS) cases are associated with dominantly inherited mutations in *SOD1* (Deng *et al*., 1993; Cudkowicz and Brown, 1996; Jeneja *et al.*, 1997). The mechanism by which single amino acid changes in the SOD molecule lead to FALS is not yet understood. Recent studies indicate that two of the human FALS mutants, A4V and G93A, catalyze the oxidation of a model substrate by hydrogen peroxide at higher rates than that seen with the wild-type enzymes (Pazos *et al*., 1996; Yim *et al*., 1996, 1997), and that in the crystal structure of the human Cu,ZnSOD mutant G37R (CuZnSOD) two SOD subunits have distinct environments in the crystals and are different in structure at their copper binding sites. Hart *et al.* (1998) suggested that the copper site asymmetry leads to loosing of the protein structure, that is the dissociation of the dimeric structure.

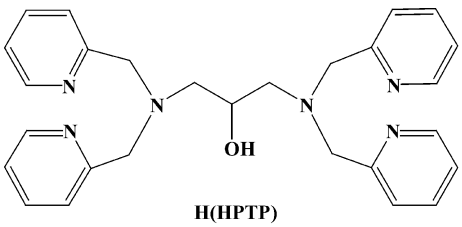
Recently Rakhit *et al*. (2004) have reported that SOD1, normally a dimeric enzyme, dissociates to monomers prior to aggregation for both wild-type and mutant proteins in the presence of copper(II) chloride and ascorbic acid by the use of DLS (dynamic light scattering). This dissociation may be accompanied by small changes in the secondary structure of SOD1, and this common intermediate suggests a common pathway for the aggregation of mutant and wild-type SOD1 providing a mechanistic link between sporadic and familial ALS. However, it remains unknown why the dimeric SOD1 dissociates into monomers in the presence of copper(II) chloride and ascorbic acid. Very recently we have reported that capillary electrophoresis (CE) is very suitable to detect the *dissociation process of the dimeric structure* of several proteins including SOD in solution (Sutoh *et al*., 2005; Chiba *et al*., 2006), and suggested that the dissociation of the SOD enzyme proceeds in the presence of hydrogen peroxide. As the formation of hydrogen peroxide has been assumed in a solution containing copper(II) chloride and ascorbic acid (Oishi *et al.*, 1980), in this study we have measured the CE profiles of the solutions containing Cu,ZnSOD in the presence of copper(II) chloride and ascorbic acid.

Materials and Methods

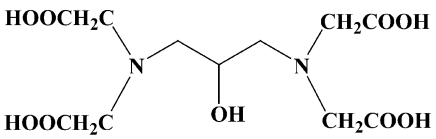
Materials

Cu,ZnSOD was purchased from Sigma (*bovine*, S2515) and other reagents were also from Sigma. The solutions consisted of 40–100 μM SOD, 4 mM ascorbic acid and 0.2 mM copper(II) chloride in 110 μl Tris [tris(hydroxymethyl)aminomethane] buffer (pH 7.3, 10 mM); the concentrations of the reagents are similar to those reported by Rakhit *et al*. (2004). Several copper(II) chelates with H(HPTP)

and H_5(HPTA) were prepared in this study; the chemical structures of these ligands are illustrated in Scheme I. H(HPTP) and H_5(HPTA) represent *N,N,N′,N′*-tetrakis(2-pyridylmethyl)-1,3-diamino-2-propanol and 1,3-diamino-2-propanol-*N,N,N′,N′*-tetraacetic acid, respectively.



H(HPTP)



H_5(HPTA)

Scheme I.

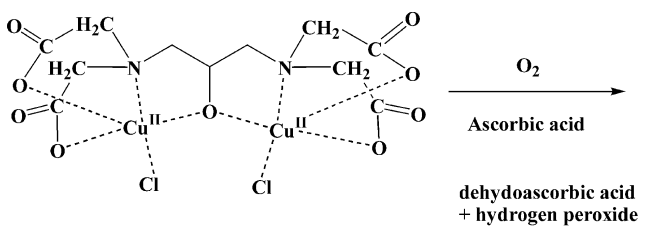
Capillary electrophoresis (CE)

Capillary electrophoregrams of the solutions were obtained with a Beckman/Coulter P/ACE MDQ instrument: temperature, 298 K; buffer solution, 10 mM Tris (pH 7.3) (Nishida *et al.*, 2007); voltage, 20 kV; uncoated column, I. D. 50 μm, 50 cm; detection, 214 nm. Two solutions, 100 μl (SOD, 3 mg/ml) and 10 μl [copper(II) chloride and ascorbic acid in Tris buffer, pH 7.3], were mixed and eluted with Tris buffer solution (pH 7.3, 10 mM).

Results

As reported in our previous paper, one sharp signal was observed in the capillary electrophoregram (~5.1 min) of a solution containing SOD (Sutoh *et al.*, 2005; Chiba *et al.*, 2006), and this can be attributed to its rigid dimeric structure. Addition of a solution of copper(II) chloride or ascorbic acid to the SOD solution did not change the CE response. On the other hand, addition of a solution containing both copper(II) chloride and ascorbic acid gave a drastic change of the capillary electrophoregram; as illustrated in Fig. 1 (B and C) a marked decrease of the peak intensity (~30%) was detected. This should be due to the

Fig. 1. Capillary electrophoregrams (A) of SOD solution, (B) measured immediately after addition of copper(II)/ascorbic acid, and (C) measured 60 min after addition of copper(II)/ascorbic acid.



Scheme II. $[Cu_2(HPTA)Cl_2]^{3-}$

dissociation of the SOD molecule to monomers (Rakhit *et al*., 2004), and this result clearly implies that the CE method is very useful to detect the dissociation of the dimeric SOD enzyme to monomers in solution.

This change in the capillary electrophoregram found above is very similar to that observed after addition of hydrogen peroxide solution to the SOD enzyme (Chiba *et al*., 2006). Since it has been pointed out that hydrogen peroxide is formed in a solution of copper(II) chloride and ascorbic acid, above facts all suggest that the dissociation of the dimeric SOD molecule into monomers should be due to the effect by hydrogen peroxide. We already reported that hydrogen peroxide is readily generated in the reaction mixture of dimeric copper(II) compounds and ascorbic acid (Scheme II) (Oishi *et al*., 1980), and this is quite consistent with the similar notable decrease of the CE peak strength due to the dimeric SOD enzyme observed when the solution of the copper(II) compound together with H(HPTP), $Cu_2(HPTP)Cl_2ClO_4$, or $H_5(HPTA)$, $Na_3Cu_2(HPTA)Cl_2$ (Scheme II) was added to a solution containing SOD and ascorbic acid.

These findings clearly suggest that the presence of hydrogen peroxide may be an potential factor to induce the sporadic ALS, and new countermeasures to prevent the sporadic ALS should be developed on the basis of the results obtained in this study (Liochev and Fridovich; 2002, Nishida, 2004, 2006).

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